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chemagic cfDNA 5k Kit

(art. No. CMG-134)

Manual Purification Protocol for 1 - 5 mL of Plasma

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Any further questions?

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Intended Use

With the **chemagic cfDNA 5k Kit** circulating cell-free DNA (cfDNA) can be isolated from 1 - 5 mL of plasma obtained from human whole blood samples. All the reagents needed for the isolation of cfDNA are included in the kit, with the exception of nuclease-free water for dissolving Proteinase K. The kit components provide sufficient material for 40 preparations.
The kit is designed to be used manually with the **chemagic** Stand 2x12 and **chemagic** Stand 50k Type B. The product is intended for professional users such as technicians and physicians trained in molecular biology techniques.

Contents of the Kit

M-PVA Magnetic Beads	1 x 10 mL	**Binding Buffer 2**	2 x 125 mL
Lysis Buffer 1	1 x 15 mL	**Wash Buffer 3**	1 x 100 mL
Proteinase K (lyophilized)	5 vials	**Wash Buffer 4**	1 x 100 mL
Poly(A) RNA (lyophilized)	1 vial	**Elution Buffer 5**	1 x 10 mL
Poly(A) RNA Buffer	1 vial		

Functional Principle

The **chemagic cfDNA 5k Kit** is based on chemagic Technology using **M-PVA Magnetic Beads** for the isolation of cfDNA. The cfDNA binds to paramagnetic beads which are magnetically separated from the sample material. During subsequent steps contaminants are removed and the purified cfDNA is transferred into an elution medium.

Quality Control

Each lot is tested to ensure the product meets the defined specifications according to chemagen's quality management system. Suboptimal results may be obtained if the protocol is not strictly followed.

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Product Specifications

The kit is designed for the use with human plasma samples derived from stabilized blood collection tubes.

The kit is adapted to a manual procedure with differences regarding incubation and time efficiency compared to the **chemagic 360** platform. For an automated extraction heat incubation is not required.

Fresh and frozen plasma can be used. Please refer to section "**Plasma Preparation Protocol**" below.

The kit is not intended for the use with whole blood or tissue sample material. The isolation efficiency of cfDNA with other sample materials has not been investigated.

All reagents required for the cfDNA isolation are included in the kit except nuclease-free water for dissolving **Proteinase K**.

The **Elution Buffer 5** included in this kit is 10 mM Tris-HCl pH 8,0 with 0,1 mM EDTA. 10 mM Tris-HCl pH 8,0 can also be used without any protocol adjustments. Water pH 8,0 may also be used, but the yield could be slightly decreased.

Required Equipment

- CMG-134, **chemagic cfDNA 5k Kit** (40 preps)
- CMG-300, **chemagic** Stand 2x12 for 1,5 mL and 2,0 mL
- CMG-303, **chemagic** Stand 50k Type B for 50 mL centrifuge tubes
- Waterbath or thermoshaker suitable for 50 mL tubes
- Vortex mixer
- Thermoshaker for 1,5 mL tubes
- Ice
- Conical 50 mL tubes, 1,5 mL tubes, 1,5 mL low binding tubes
- Centrifuge for 50 mL tubes (optional)
- Mini centrifuge for 1.5 mL tubes (optional)

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Stability and Storage

The shelf life of the kit is 18 months. Expiry dates are noted on the kit label and on the labels of the individual kit components. All components of the unused kit can be stored at room temperature. Do not use the kit beyond the expiry date.

Once the kit has been opened, the “in use” stability of the kit is 3 months. All **Wash Buffers**, **Lysis Buffer 1**, **Binding Buffer 2, Poly(A) RNA Buffer** and the **M-PVA Magnetic Beads** of the “in use” kit can be stored at room temperature.

Binding Buffer 2, **Wash Buffer 3** and **Wash Buffer 4** contain ethanol. Longer storage of the buffers without lids should be avoided. If ethanol evaporates the optimal yield cannot be guaranteed.

Store **Poly(A) RNA Buffer, Binding Buffer 2** and **Wash Buffer 3** in the dark.

Lysis Buffer 1 may form a precipitate upon storage. If necessary, warm up to 55 °C to dissolve.

The reconstituted **Poly(A) RNA** in **Poly(A) RNA Buffer** and reconstituted **Proteinase K** are stable for 4 weeks at 4 °C.

For long-term storage we recommend to store the reconstituted **Poly(A) RNA** in **Poly(A) RNA Buffer** and **Proteinase K** in aliquots at -20 °C. Do not freeze the **Poly(A) RNA** in **Poly(A) RNA Buffer** and **Proteinase K** aliquots after thawing.

Before using equilibrate **Poly(A) RNA** and **Proteinase K** to room temperature.

The use of **Poly(A) RNA** is recommended as indicated in the detailed protocol description below. The extraction of cfDNA using this kit is possible without the use of **Poly(A) RNA** however the quantification of cfDNA may vary.

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Quantification Methods – Comments

In some cases you may find traces of **M-PVA Magnetic Beads** remaining in the eluate. In such a case we recommend a short centrifugation of the samples to isolate the remnant **M-PVA Magnetic Beads** at the bottom of the vessel, or perform an additional separation step using an appropriate **chemagic** magnetic stand in order to separate traces of particles.

During development the performance of the automated kit was evaluated using the following quantification methods:

- qPCR on ALU115 primer set[1] on the QuantStudio® 5 Real-Time PCR System
- Qubit™ 1X dsDNA HS Assay Kit on the Qubit® 3.0 Fluorometer

cfDNA yields isolated from human plasma samples are typically in the range of 1 - 30 ng/mL of plasma and therefore critically low and maybe outside the detection parameters determined by spectrophotometric methods.

If quantification of the extracted cfDNA is required a PCR-based method (qPCR, ddPCR) is recommended.

When using fluorometric quantification methods the addition of **Poly(A) RNA** is essential for a reliable performance. Fluorometric analysis of eluates extracted without the use of **Poly(A) RNA** may lead to varying and decreased quantification data. Additionally, be careful in interpreting the results if fluorometric methods are used for quantification as not only cfDNA but the total DNA is measured.

Qualitative Methods – Comments

During development the performance of this kit was evaluated using the following qualitative method:

- DNA NGS 3K Assay with the LabChip® GX Touch/GXII Touch

To assess fragment distribution of the extracted cfDNA, fragment analysis systems may be used. A major peak at around 150 - 170 bp is expected for high quality mononucleosomal cfDNA, in some cases a smaller peak at around 300 bp representing dinucleosomal cfDNA fragments is also present.

The use of fragment analysis systems for cfDNA quantification did not lead to reproducible results during kit development and is not recommended. Marker peak areas (marker included in fragment

[1] ALU115 primer sequences obtained from: T.B. Hao, British Journal of Cancer (2014) 111, 1482–1489

analyzer kits) vary according to different extraction chemistries. Variations of marker peak areas within one fragment analysis run may result in miscalculations.

Plasma Preparation Protocol

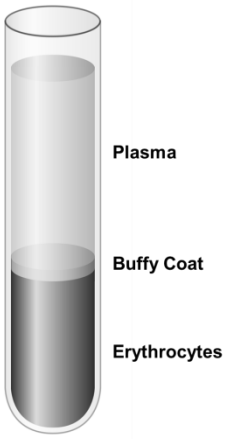
It is recommended to prepare plasma as fresh as possible (max. 5 days after blood draw). Longer storage of blood prior to plasma preparation may lead to poor separation results and higher background from genomic DNA.

A double centrifugation protocol during plasma preparation is recommended to minimize the potential for contamination of plasma with cells and genomic DNA. Transfer of any other blood components (buffy coat or red blood cells) should be avoided while separating the plasma fraction.

Please refer to the tube manufacturers specifications (max. centrifugation speed) for more information.

From 10 mL of whole blood approximately 4 - 5 mL plasma can be expected.

General Plasma Preparation Protocol



1. Centrifuge whole blood collection tube at 2.000 x g for 20 minutes.
2. Aspirate plasma carefully and at least 2 - 3 mm above the buffy coat layer, without disturbing the layer and transfer it into a new appropriate tube.
3. Centrifuge the plasma sample at 3.300 x g for 30 minutes.
4. Carefully transfer the supernatant to a fresh tube without disturbing the pellet, at least 2 - 3 mm above the pellet.
5. For direct use, storage of plasma sample at 2 - 8 °C for up to 10 hours is possible, for long-term storage -80 °C is recommended.
6. Before cfDNA extraction, equilibrate plasma to room temperature. Thaw the frozen plasma storage tubes at room temperature for approximately 30 minutes (until thawed) or using a water bath (25°C to 30°C) for 10 minutes. Ensure that the tubes are thoroughly thawed before cfDNA extraction is started. If there are precipitates in the thawed plasma, dissolve them by inverting the tubes.

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Processing Steps in Detail

Before You Start

- Dissolve lyophilized **Poly(A) RNA** by adding 440 µL of **Poly(A) RNA Buffer** to the **Poly(A) RNA** tube and mix thoroughly.
- Dissolve lyophilized **Proteinase K** in 1,25 mL nuclease-free water.
- Preheat water bath or thermoshaker to 55°C.

! ***See "Stability and Storage"***

- A homogenous suspension of the **M-PVA Magnetic Beads** must be ensured to guarantee the correct **M-PVA Magnetic Bead** concentration. Mix the bottle containing the **M-PVA Magnetic Beads** vigorously and check the bottom of the bottle for **M-PVA Magnetic Beads** sedimentation before dispensing. Otherwise optimal cfDNA extraction performance cannot be ensured.

The following table lists volumes of the components to be used, depending on starting plasma sample volume.

Component	Plasma Sample				
	1 mL	2 mL	3 mL	4 mL	5 mL
Poly(A) RNA	1,3 µL	1,3 µL	1,3 µL	1,3 µL	1,3 µL
Proteinase K	100 µL	100 µL	150 µL	150 µL	150 µL
Lysis Buffer 1	50 µL	100 µL	150 µL	200 µL	250 µL
M-PVA Magnetic Beads	30 µL	60 µL	90 µL	120 µL	150 µL
Binding Buffer 2	1,25 mL	2,5 mL	3,75 mL	5,0 mL	6,25 mL
Drying Step	10 min at room temperature or 2 min at 55 °C		15 min at room temperature or 4 min at 55 °C		
Elution Buffer 5	30 - 60 µL		50 - 100 µL		

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Protocol Steps

1. Prefill the 50 mL sample tube with the indicated volume of **Proteinase K** and 1,3 µL dissolved **Poly(A) RNA**, add the plasma sample equilibrated to room temperature and **Lysis Buffer 1**. Vortex briefly for **10 seconds** and incubate the sample for **10 minutes** at 55 °C in a water bath or heating block.

   Poly(A) RNA, **Proteinase K** and **Lysis Buffer 1** can be premixed by transferring **Proteinase K** to an appropriate vessel first and then adding **Poly(A) RNA** followed by the **Lysis Buffer 1** (choose the appropriate volume of **Poly(A) RNA** / **Proteinase K** / **Lysis Buffer 1** to ensure you have a sufficient amount for the number of isolations).

The Proteinase K activity will decrease after incubation longer than 10 minutes in Lysis Buffer 1. Ensure that all samples are mixed with Poly(A) RNA / Proteinase K / Lysis Buffer 1 within this time.

2. Following lysis incubation, cool the tubes containing the plasma sample to room temperature by placing them on ice for **5 minutes**.
3. Add the indicated volume of **M-PVA Magnetic Beads** and **Binding Buffer 2** to each sample. Mix briefly and incubate for **10 minutes** with continuous agitation by vortexing or on a thermoshaker at room temperature.
4. Following binding incubation, place the tube on **chemagic Stand 50k Type B** for **5 minutes** or until the solution clears and **M-PVA Magnetic Beads** are completely separated. Thoroughly remove any foam and discard the supernatant.

   Optional: Briefly centrifuge the lysis/ binding tube to remove any foam and to separate M-PVA Magnetic Beads pellet. Keep the tube on the magnet for another minute and remove the residual supernatant.
5. Resuspend M-PVA Magnetic Beads in 1 mL of Wash Buffer 3, then transfer **M-PVA Magnetic Beads/DNA Complex** to a 1,5 mL tube and save the lysis/binding tube.
6. Place the 1,5 mL tube containing the **M-PVA Magnetic Beads/DNA Complex** on the **chemagic Stand 2x12** (magnet position) for 30 seconds, collect the **Wash Buffer 3** supernatant and rinse the lysis/binding tube.
7. Transfer any residual beads to the tube containing the **M-PVA Magnetic Beads/DNA Complex** and discard the lysis/binding tube.
8. Separate the **M-PVA Magnetic Beads/DNA Complex** in a **chemagic Stand 2x12** (magnet position) for **2 minutes**. Aspirate and discard supernatant.

9. Remove tube from the magnetic separator and add 1000 µL **Wash Buffer 3** to the tube and thoroughly resuspend the beads in **Wash Buffer 3** by pipetting the bead pellet up and down 15 times or by vortexing **30 seconds**.
10. Separate the **M-PVA Magnetic Beads/DNA Complex** in a **chemagic Stand 2x12** (magnet position) for **1 minute**. Aspirate and discard supernatant.

! ***Take care that there is no residual liquid and bead slurry left in the lid, alternatively liquid can be shortly spun down.***

11. Remove tube from the magnet position and repeat the washing procedure twice (steps 9 and 10) using 1000 µL **Wash Buffer 4**.
12. After removing the last traces of the second **Wash Buffer 4** step, leave tube in the magnet position and dry the bead pellet for **10 - 15 minutes** at room temperature or **2 - 4 minutes** at 55 °C on a thermoshaker (see table in section "***Before You Start***").

! ***Take care that the bead pellet is dried, extend drying time by another 1 – 2 minutes if necessary.***

13. Add 30 - 100 µL of **Elution Buffer** to the tube and thoroughly resuspend the **M-PVA Magnetic Beads/DNA Complex** by pipetting the pellet up and down 10 to 15 times.
14. Incubate the suspension for **5 minutes** at room temperature, with occasional agitation to facilitate complete DNA elution.

    Optional: Incubate the suspension **5 minutes** at 55 °C with gentle agitation. In some cases this may increase the final yield.

15. Following DNA elution, place the tube in a **chemagic Stand 2x12** (magnet position) for **2 minutes** or until all the **M-PVA Magnetic Beads** have separated from the eluate. Transfer the **eluate** containing the purified DNA to a clean low binding tube.

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Safety Information

To avoid injuries working with the kit components always wear safety glasses, disposable gloves and protective clothing. For detailed information please refer to the according Safety Data Sheet.

Reagent 1: **M-PVA Magnetic Beads** No hazardous substances in reportable concentrations, which must be labeled.
Reagent 2: **Lysis Buffer 1** **Substance(s):** Sodium dodecyl sulfate 20% ($CH_3(CH_2)_{11}OSO_3Na$) **CAS No.:** 151-21-3 ($CH_3(CH_2)_{11}OSO_3Na$)
Reagent 3: **Binding Buffer 2** **Substance(s):** Guanidine thiocyanate 35 - 45% ($C_2H_6N_4S$), dissolved in ethanol 20 - 40% (EtOH) **CAS No.:** 64-17-5 (EtOH), 593-84-0 ($C_2H_6N_4S$)
Reagent 4: **Wash Buffer 3** **Substance(s):** Guanidine thiocyanate 15 - 25 % ($C_2H_6N_4S$), dissolved in ethanol 30 - 50% (EtOH) **CAS No.:** 64-17-5 (EtOH), 593-84-0 ($C_2H_6N_4S$)
Reagent 5: **Wash Buffer 4** Substance(s): aqueous ethanol solution 70 - 90% (C_2H_5OH) **CAS No.:** 64-17-5 (EtOH)
Reagent 6: **Elution Buffer 5** No hazardous substances in reportable concentrations, which must be labeled.
Reagent 7: **Poly(A) RNA Buffer** **Substance(s):** Guanidine thiocyanate 30 - 40%($C_2H_6N_4S$) **CAS No.:** 593-84-0 ($C_2H_6N_4S$)
Reagent 8: **Poly(A) RNA** No hazardous substances in reportable concentrations, which must be labeled.

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